PAN-PACIFIC ENTOMOLOGIST
72(3): 121–129, (1996)

POSSIBLE ROLE OF NONFERTILIZING SPERM AS A NUTRIENT SOURCE FOR FEMALE *DROSOPHILA PSEUDOOBSCURA* FROLOVA (DIPTERA: DROSOPHILIDAE)

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Abstract.—Males in some insect taxa produce chromosomally or morphologically variant sperm types, one of which does not fertilize eggs. Hypotheses as to the functional significance of nonfertilizing sperm in these taxa state that nonfertilizing sperm may represent nutrient resources utilized by fertilizing sperm, the female mate or the zygote. *Drosophila pseudoobscura* Frolova males produce two discrete lengths of sperm, short and long, but short sperm do not participate in fertilization. Additionally, females of this species incorporate ^{14}C male-derived materials into their tissues. We tested whether these male-derived substances incorporated by *D. pseudoobscura* females originate from nonfertilizing short sperm and could represent a nutrient donation by males. We tracked the fate of ^{14}C material from the male within female tissues and found that females incorporated radiolabel into somatic tissues by six hours after copulation. However, short sperm do not begin to disappear from sperm storage organs until 6 hours after copulation and we found no association between the subsequent loss of short sperm in storage and the amount of male-derived material consequently incorporated into female somatic tissues or oocytes. These results suggest that short nonfertilizing sperm are not the source of ^{14}C male-derived components incorporated by females and we conclude that short sperm do not serve as nutrient donations to the female mate or the zygote.

Key Words.—Insecta, nonfertilizing sperm, male-derived nutrient donations, *Drosophila pseudoobscura,* sperm polymorphism, ejaculate

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Males in certain insect taxa transfer products at mating that 1) become incorporated into female somatic tissues and ovarian oocytes (e.g., Boggs & Gilbert 1979, Thornhill & Alcock 1983, Markow & Ankney 1988) and, 2) are associated with increases in fitness components of the female mate or zygote (Gwynne 1981, 1988; Butlin et al. 1987; Rutowski et al. 1987). In those *Drosophila* species in which females incorporate ejaculatory materials, it has been assumed that the incorporated material is derived from male accessory gland products, but this assumption has not been tested directly (Bownes & Partridge 1987, Markow & Ankney 1988, Pitnick et al. 1991). Sperm, not accessory gland secretions, could potentially function as male-derived nutrient resources. In species in which males simultaneously produce two sperm types, one of which functions in fertilization and one that cannot fertilize eggs, nonfertilizing sperm could be nutritive. Such nutrients may benefit the fertilizing sperm types, the female mate or the zygote (Hanson et al. 1952, Sivinski 1980, Healy & Jamieson 1981, Silberglied et al. 1984).

Males of species in the *Drosophila obscura* Fallén group produce two distinct lengths of nucleated sperm, short and long, within each ejaculate (Beatty & Sidhu 1970). We have found that, in *D. pseudoobscura* Frolova, only long sperm morphs

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fertilize eggs despite each sperm type being transferred in equal proportions to females (Snook et al. 1994). Short sperm begin to move into sperm storage organs (SSOs) approximately two hours after copulation, but between 6 and 12 h post-mating the number and proportion of short sperm begins to decrease in SSOs while the number and proportion of long sperm continues to increase during these times. These changes in the numbers of each sperm type found in SSOs do not alter the proportion of short or long sperm found in the uterus until oviposition begins. The mechanism of "disappearance" of short nonfertilizing sperm from female SSOs is unknown but perhaps these sperm could be broken down and serve as the source of male-derived ^{14}C material incorporated into female tissue. It is unlikely that short sperm within the uterus serve this function since the proportion of short sperm found there does not change until oviposition begins 24 to 48 h after mating (Snook et al. 1994).

To test if male-derived material in female *D. pseudoobscura* is from nonfertilizing sperm that could then serve as a beneficial nutrient donation, we examined the timing of incorporation of male-derived substances into female tissues. We predicted that if short, nonfertilizing sperm function as nutrient resources, the temporal decrease of short sperm within female SSOs beginning between 6 and 12 h after mating (Snook et al. 1994) should correspond to increasing amounts of male-derived substances detectable in female tissues.

Materials and Methods

Fly cultures.—Drosophila pseudoobscura was established from flies collected on fallen citrus in Tempe, AZ in 1990 and 1991. Flies were maintained on standard cornmeal-agar-molasses food with yeast and kept at room temperature, 22–25° C, and an approximate 12:12 L:D photoperiodic cycle. All flies used in experiments were 5 days old and reproductively mature.

Tracking ejaculate contributions.—If short sperm function as a nutrient source, their disappearance should precede the detection of male-derived materials in female tissue. To track the timing of male-derived material in females, we mated females to radiolabelled males and assayed radioactivity subsequently found in female tissues at 6, 12, and 24 h after copulation, corresponding to prior times examining sperm movement in SSOs (Snook et al. 1994). Fifty, first instar larvae from culture bottles were placed into vials containing 5 g of culture media inoculated with 50μCi of a ^{14}C labelled mixture of amino acids. Virgin males were collected from this larval culture on eclosion and stored 10 per food vial with dry yeast until they were mated to nonradiolabelled, virgin females. These females were nutritionally stressed because previous work noted that the greatest effect of male substances on females occurred when females were food-limited (Bownes & Partridge 1987, Butlin et al. 1987, Markow et al. 1990, Fox 1993; but see Chapman et al. 1994). Females were collected upon eclosion, stored in food vials (10/vial), and nutritionally stressed by providing no surface yeast in the food vials.

Two virgin radiolabelled males were placed with one nutritionally stressed virgin female in an unyeasted food vial and upon mating, the "unsuccessful" male was aspirated from the vial without disturbing the copulating pair. Immediately after copulation ended the successful male was removed from the vial. Nine females were examined at each time interval (6, 12 and 24 h) after mating. They

were ether-anesthetized and processed for scintillation counting to determine the amount of radioactivity present.

To process mated females for scintillation, flies were washed once for 30 sec in a microcentrifuge tube containing a mixture of phosphate buffer solution (PBS) and Triton-X to remove any external radiolabel transferred by the male during copulation, followed by another wash in PBS after which they were decapitated (eye pteridines contribute to a quenching effect). Female reproductive tracts were removed intact and the ovaries detached from the common oviduct. The remainder of the reproductive tract from each female was examined for the presence of sperm, and ovaries and the remaining somatic tissue were washed separately as described above for whole flies. Ovarian and somatic tissue samples from three females were pooled in a scintillation vial containing tissue solubilizer. Tissues were then crushed with the end of a clean glass rod and digested for 24 h in a 50° C waterbath. At room temperature, the tissues were first neutralized with glacial acetic acid, followed by the addition of scintillation fluid. Subsequently, tissues were vortexed, and 24 h later samples were counted by a Beckman LS 7000 liquid scintillation system. Counts per minute were converted to disintegrations per minute following a standard quench curve (Pitnick et al. 1991). Nutritionally stressed females mated to nonradiolabelled males ($n = 9$) served as a control and were processed as above. Scintillation vials containing no tissue ($n = 6$) were used to determine background counts.

Statistical analyses.—To test the prediction that the disappearance of short sperm within SSOs corresponds to an increase in radiolabel detected in other tissues of females, we compared data originally reported in Snook et al. (1994), describing the number of short sperm found in SSOs at 6, 12, and 24 h after mating, with the amount of radiolabel found in females at these times. We generated the mean ± standard error (SE) of short sperm found in SSOs at 6, 12, and 24 h after mating, by pooling data from the ventral receptacle and spermathecae of nonovipositing females. One datum collected at 24 h after mating was an extreme outlier and was removed from analyses; the number of short sperm found in the ventral receptacle of this sample was approximately 1000 while the mean ± SE of the remaining seven samples was 91.7 ± 36.6.

Radiolabel data and sperm count data were acquired during different experiments. However, reported sperm counts for each time examined in Snook et al. (1994) were replicated 3 times over a 2 month period with the entire dataset being collected over 10 months. Despite the temporal disparity in when replications were collected, there were no significant differences in the numbers of short sperm found among replicates in each sperm storage organ at 12 and 24 h after mating (replicates were tested using Kruskal-Wallis ANOVA because values were not normally distributed (Sokal & Rohlf 1981); 12 h (total n over all replicates = 13), ventral receptacle (VR), $P = 0.34$, spermathecae (SP), $P = 0.38$; 24 h (total n over all replicates = 7) VR, $P = 0.13$, SP, $P = 0.24$)). At 6 h after mating, however, there were significant differences among replicates in the number of short sperm found in the VR ($P = 0.01$), but not in the SP (total n over all replicates = 14, $P = 0.183$). The difference in the number of short sperm found in replicates of the VR seemed to be due to one replicate in which there were smaller numbers of both short and long sperm found. Subsequently, we tested if the *proportion* of short sperm differed between replicates; there was no difference

(n = 14; P = 0.12; Snook et al. 1994). Therefore, the pattern of sperm storage and the amount, either the number or proportion of short sperm, found in SSOs are highly constant between females and throughout time, justifying the use of these earlier storage data in testing our prediction. All statistics were performed using Systat (version 5.03, Systat, Inc; Wilkinson 1990).

Results

The proportion of short sperm in the uterus does not change from the time of transfer until more than 24 h after copulation (F = 1.339; df = 4, 56; P = 0.267; Fig. 1). However, comparing the number of short sperm found in SSOs between 6 to 24 h postmating indicated that short sperm "disappeared" within SSOs (F = 4.933; df = 2, 31; P = 0.01; Fig. 1). Tukey's *a posteriori* tests indicated that significant disappearance of short sperm occurred between 6 and 12 h (P = 0.037) and between 6 and 24 h (P = 0.042) but not between 12 and 24 h ($P > 0.05$). The proportion of short sperm found in SSOs also decreased over time (F = 6.355, df = 2, 31; P = 0.005). However, only the comparison between 6 and 24 h was significant (P = 0.005). The number of sperm found in SSOs 2 h after mating is reported in Fig. 1 to indicate that the maximum number of short sperm is stored at 6 h postcopulation. Comparing the number of long sperm found in SSOs between 6 to 24 h postmating indicated that long sperm do not continue to significantly increase (F = 2.933, df = 2, 31, P = 0.07; Fig. 1).

If short sperm serve as a nutrient contribution, then the increase in incorporation of radiolabel in female tissues is predicted to occur subsequent to 6 h postcopulation, when short sperm are maximally stored. We found that significant amounts of radiolabel were incorporated in the somatic tissue (F = 9.284; df = 3, 8; P = 0.006; Fig. 2), but not the oocytes (F = 3.115; df = 3, 8; P = 0.09), of starved *D. pseudoobscura* females. Contrary to our prediction, however, Tukey's *a posteriori* tests indicated that significant radiolabel incorporation occurred between 0 h and 6 h (P = 0.035) after mating, but there was no significant increase after 6 h (Fig. 2).

Discussion

The evidence presented here indicates that short sperm do not function as a nutrient resource for *D. pseudoobscura* females. If short nonfertilizing sperm in *D. pseudoobscura* break down and function as a nutrient source, the surge in the amount of radiolabel detected in female tissues should have occurred when the short sperm begin to disappear from the SSOs, between 6 h and 12 h after copulation (Fig. 1; Snook et al. 1994). The increase in radiolabel in somatic tissues that we observed was prior to 6 h, when the number of short sperm was the largest within SSOs. Short sperm in the uterus are also unlikely to serve as nutrient donations because the proportion of short sperm does not change from the

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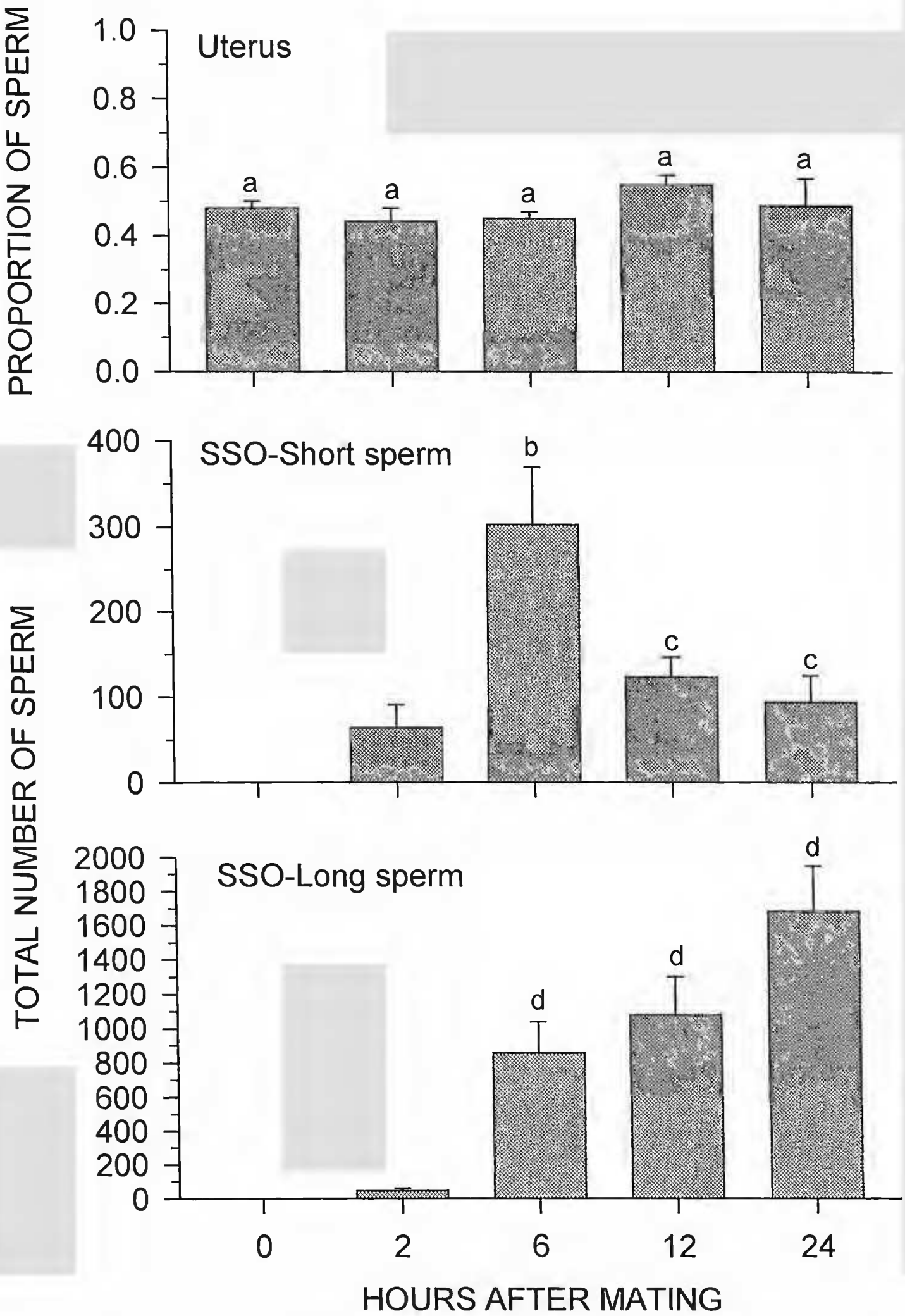
Figure 1. Mean (±SE) of the proportion of short sperm present in the uterus and the total number of short and long sperm in the sperm storage organs (SSOs-ventral receptacle and spermathecae) of *D. pseudoobscura* at various times after mating. Values at 0 h indicate sperm in the uterus immediately after copulation and thus, represent male sperm transfer. Females do not have sperm in SSOs at 0 h



postmating. Sample sizes are 11 for 0 h, 15 for 2 h uterus, 13 for 2 h SSOs, 15 for 6 h, 13 for 12 h, 7 for 24 h. Values for 24 h are for females that have not oviposited. Letters a–d denote significantly different means detected by Tukey's *a posteriori* multiple comparison tests; comparisons for the uterus (letter a), short sperm in the sperm storage organs (SSO-Short sperm) (letters b and c), and long sperm in the sperm storage organs (SSO-Long sperm) (letter d) were tested separately.

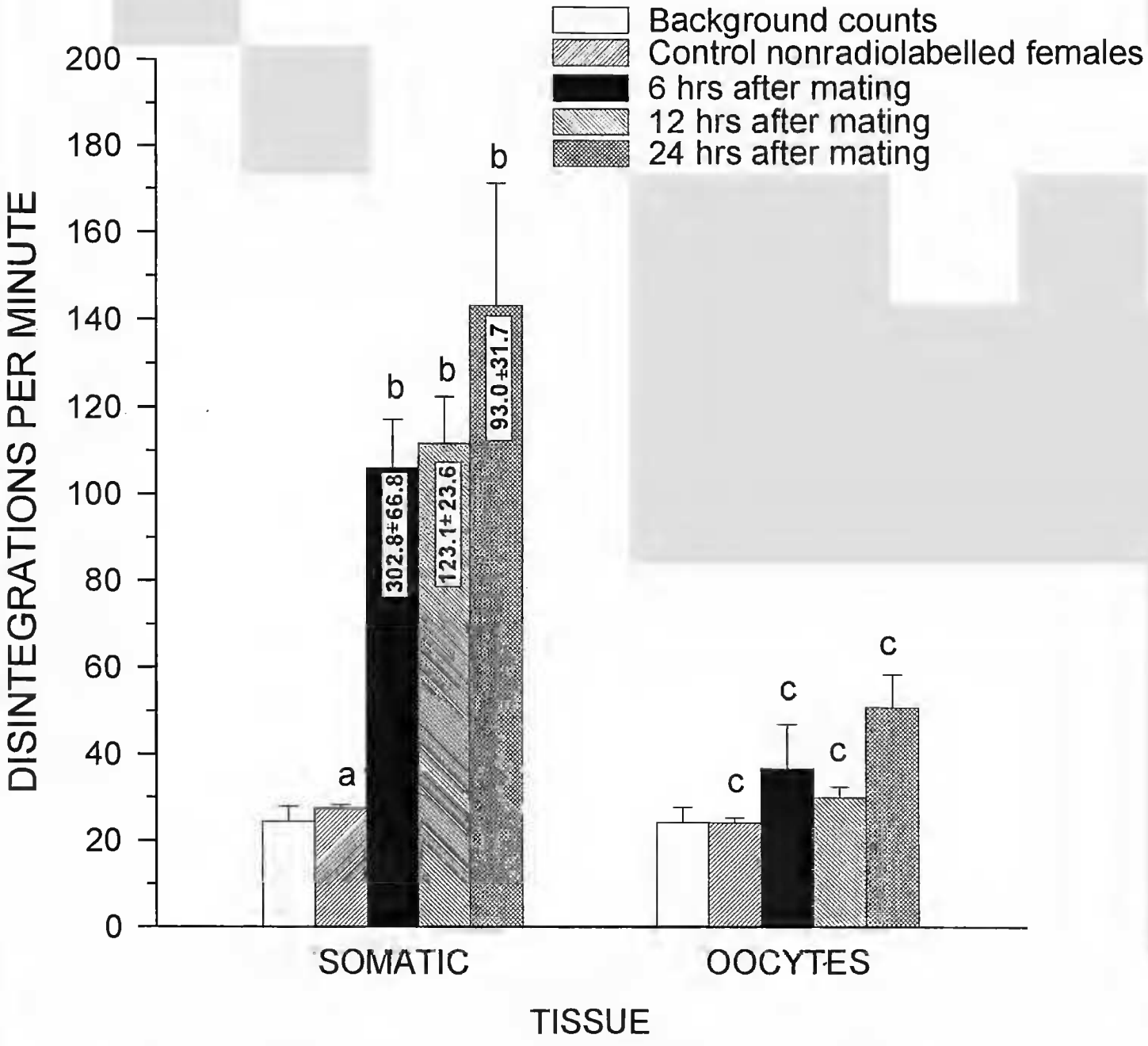


Figure 2. Mean (±SE) disintegrations per minute of radiolabel found in female somatic and ovarian tissue 6, 12, and 24 h after mating to radiolabelled males compared to nonradiolabelled controls. Numbers within bars represent the number of short sperm ± SE found in sperm storage organs at these times after copulation (from Snook et al. 1994; see also text). Letters a–c denote significantly different means detected by Tukey's *a posteriori* multiple comparison tests; comparisons for somatic tissue (letters a and b) were tested separately from oocytes (letter c).

time of transfer until more than 24 h after copulation when oviposition begins. The numbers of sperm in the uterus were not determined due to the large amount of sperm transferred by males to females. Thus, our data indicate that although nonfertilizing sperm have been suggested to perform an adaptive role as nutrient resources to fertilizing sperm, the female mate or the zygote (Hanson et al. 1952, Healy & Jamieson 1981, Sivinski 1980, Silberglied et al. 1984), in *D. pseudoobscura* nonfertilizing short sperm do not function in this manner. Rather than products from the potential breakdown of short sperm, the origin of ejaculatory contributions to females are nonsperm accessory gland materials.

One assumption in arriving at the above conclusion is that active transport or exocytosis of labelled materials occurs across the intact sperm cell. To our knowledge no evidence in *Drosophila* exists for or against specific transport mechansims on sperm membranes. Movement of materials across short sperm could occur prior to our detecting their "disappearance" as a result of breaking down. How-

ever, the mechanism contributing to the disappearance of short sperm from SSOs in *D. pseudoobscura* is unknown. The decrease in short sperm found in SSOs may not be a result of these sperm types breaking down but of being pushed out from storage by long fertilizing sperm, a mechanism currently being tested.

The location of radiolabel incorporation that we found differs from two earlier reports in that we found significant incorporation into somatic rather than ovarian tissues (Bownes & Partridge 1987, Markow & Ankney 1988). The "ovarian" tissues examined by Bownes & Partridge (1987) may have included other portions of the female reproductive tract (uterus, ventral receptacle and spermathecae) that contained radiolabelled sperm and accessory gland secretions. This discrepancy in protocol between studies prevents any direct comparison of the differences in results. Markow & Ankney (1988) used the same dissection procedures as in this report, but utilized females that were well-fed, not starved. The nutritionally stressed females in this report had few mature oocytes in comparison to well-fed females (Snook, personal observation). Nutritional stress may cause competition for the same resource between reproduction and survival, resulting in trade-offs between these functions (Stearns 1992). Females in poor condition may allocate energy into somatic maintenance, rather than reproduction, because oogenesis requires substantial resources (Robertson & Sang 1944, Sang & King 1961). This tradeoff may explain the difference between prior results (Markow & Ankney 1988) and ours. We conclude that patterns of utilization of male-derived substances appear to be influenced by the physiological condition of the female.

That tradeoffs occur are supported by the observation that females regulate oviposition behavior based on their nutritional status with increased oviposition rates associated with better nutritional status (Sang & King 1961, Chapman et al. 1994). Moreover, Bownes & Partridge (1987) demonstrated that nutritionally stressed females incorporated a greater percentage of radiolabel in somatic tissues compared to well-fed females (somatic tissues examined were not affected by potential contamination of radiolabelled sperm or accessory gland material). Other *obscura* group species also show nutrient limitations on female reproduction. Male *D. subobscura* Collin present an oral drop of liquid to females as part of courtship behavior (Brown 1956a, b; Spieth 1966, 1978) and females denied access to male drops have reduced fecundity (Steele 1986). Males of *D. pseudoobscura* also present drops to females (Steele 1986) but only if other courtship behaviors have failed (Brown 1956a, b). The effect of these drops on the reproductive fitness of *D. pseudoobscura* females is unknown. However, Turner & Anderson (1983) found that nutritionally stressed *D. pseudoobscura* females given continual access to males had a relatively greater increase in productivity compared to nutritionally stressed females only briefly exposed to males. Males could have improved the productivity of these females through either oral drops or ejaculatory secretions. Whether uptake of such substances by *Drosophila* females increases fecundity or survival is debated (Markow et al. 1990, Chapman et al. 1994) but is likely to be a species specific response based on ecology and other reproductive behaviors of a particular species. Seminal products have increasingly been found to influence female behavior and mortality (Chen 1984, Kalb et al. 1993, Chapman et al. 1994, 1995), sperm storage (Perotti 1971), and the outcome of sperm competition (Harshman & Prout 1994, Clark et al. 1995) suggesting their importance irrespective of use as nutrient contributions.

The *obscura* group has evolved several mechanisms of male donation to females, including ejaculatory contributions (through accessory gland secretions) and courtship feeding. However, the production and transfer of short nonfertilizing sperm, at least measured by ^{14}C, is not among them. The role of nonfertilizing sperm may be to function in sperm competition by giving first or second males a fertilization advantage (Sivinski 1980, Silberglied et al. 1984, Snook 1995). These alternatives are currently being tested.

Acknowledgment

We thank Scott Pitnick for help with the radiolabel dissections and John Alcock, Christine Boake, Kathy Church, Thomas Dowling, and Michael Moore and two anonymous reviewers for comments on prior versions of the manuscript. Research presented was supported by a NSF Dissertation Improvement Grant (DEB-9224263) to R.R.S.

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Received 1 Nov 1995: Accepted 14 Feb 1996